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Identification of Novel Imprinted Transcripts in the Prader-Willi Syndrome and Angelman Syndrome Deletion Region: Further Evidence for Regional Imprinting Control

Syann Lee and Rachel Wevrick

Department of Medical Genetics, 842 Medical Sciences Building, University of Alberta, Edmonton, Alberta, Canada

Deletions and other abnormalities of human chromosome 15q11-q13 are associated with two developmental disorders, Prader-Willi syndrome (PWS) and Angelman syndrome (AS). Loss of expression of imprinted, paternally expressed genes has been implicated in PWS. However, the number of imprinted genes that contribute to PWS, and the range over which the imprinting signal acts to silence one copy of the gene in a parent-of-origin–specific manner, are unknown. To identify additional imprinted genes that could contribute to the PWS phenotype and to understand the regional control of imprinting in 15q11-q13, we have constructed an imprinted transcript map of the PWS-AS deletion interval. The imprinting status of 22 expressed sequence tags derived from the radiation-hybrid human transcript maps or physical maps was determined in a reverse transcriptase–PCR assay and correlated with the position of the transcripts on the physical map. Seven new paternally expressed transcripts localize to an ~1.5-Mb domain surrounding the *SNRPN*-associated imprinting center, which already includes four imprinted, paternally expressed genes. All other tested new transcripts in the deletion region were expressed from both alleles. A domain of exclusive paternal expression surrounding the imprinting center suggests strong regional control of the imprinting process. This study provides the means for further investigation of additional genes that cause or modify the phenotypes associated with rearrangements of 15q11-q13.

Introduction

Contiguous gene syndromes involve either the duplication or deletion of a series of adjacent genes (Budarf and Emanuel 1997). Human chromosome 15q11-q13 is prone to chromosomal abnormalities, including deletions, uniparental disomy, triplication, and translocations; it is also prone to the formation of supernumerary chromosomes (Robinson et al. 1998). Two human genetic disorders, Prader-Willi syndrome (PWS [MIM 176270]) and Angelman syndrome (AS [MIM 105830]) result from rearrangements of 15q11-q13 (Nicholls et al. 1998). PWS is a neurobehavioral disorder characterized by neonatal hypotonia, developmental delay, and hyperphagia (Holm et al. 1993). Approximately 70% of affected individuals have a microscopic cytogenetic deletion of their paternal 15q11-q13 region, whereas the remainder have maternal uniparental disomy, submicroscopic deletions, or other rearrangements. Chromosome 15 deletions in PWS occur exclusively on the paternally inherited chromosome, suggesting that the genes responsible are imprinted and expressed only from the paternal allele. AS, characterized by severe developmental delay and ataxia, results from absence of expression of *UBE3A* through a maternal interstitial deletion, paternal uniparental disomy, mutations to an imprinting center (IC), or structural mutations in the *UBE3A* gene (Kishino et al. 1997; Matsuura et al. 1997). The parent-of-origin dependence of the phenotype is thought to reflect the uniparental pattern of expression of genes in the 15q11-q13 region, a phenomenon known as genomic or gametic imprinting (Tilghman 1999). In the PWS-AS region, this imprinting is proposed to be controlled by an IC (Horsthemke 1997). Specific mutations to the IC result in the silencing of multiple paternally expressed genes and result in the PWS phenotype when paternally inherited. Mutations in a nearby but distinct IC region cause AS when maternally inherited (Sutcliffe et al. 1994; Saitoh et al. 1996; Buiting et al. 1998).

Only 10 well-characterized genes have been localized to the ~4 Mb region commonly deleted in PWS and AS, although a region of this size may be expected to contain ~100 genes, a figure we based on estimates of average gene density (Fields et al. 1994; Schuler et al. 1996). Four imprinted, paternally expressed genes, *ZNF127*, *NDN*, *SNURF/SNRPN,* and *IPW,* have been localized to the centromeric end of the deletion region (Özçelik

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Address for correspondence and reprints: Dr. Rachel Wevrick, Department of Medical Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2H7. E-mail: rachel.wevrick@ualberta.ca

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et al. 1992; Wevrick et al. 1994; Jay et al. 1997; MacDonald and Wevrick 1997; Sutcliffe et al. 1997; Gray et al. 1999; Jong et al. 1999; fig. 1).

UBE3A was localized to a region telomeric to the paternally expressed cluster of genes and was originally shown to be biallelically expressed in cultured cells (Nakao et al. 1994). *UBE3A* expression has since been demonstrated to be strongly biased toward expression of the maternal allele in regions of the brain; it has been shown to be present at reduced levels in AS brain (Rougeulle et al. 1997; Vu and Hoffman 1997). Two nonimprinted genes, *P* (Rinchik et al. 1993; Lee et al. 1994) and *HERC2* (Ji et al. 1999), the latter of which is homologous to the MN7 family of repeats, are located at the telomeric end of the deleted region. Three genes with unclear imprinting status are the γ-butyric acid receptor genes, *GABRB3, GABRA5,* and *GABRG3*, located toward the telomeric part of the deletion interval (Meguro et al. 1997; Gabriel et al. 1998). The 15q11-q13 region also contains the paternally expressed transcripts PAR-1 and PAR-5 (Sutcliffe et al. 1994), the nonimprinted transcript PAR-2 (Nakao et al. 1994), and two low-level transcripts of unknown imprinting status, PAR-4 and PAR-7 (Sutcliffe et al. 1994). Repeated sequences with high sequence similarity to each other are located at either end of the common-deletion region. They predispose the PWS-AS region to deletions (Amos-Landgraf et al. 1999; Christian et al. 1999). The recently localized but not well-characterized *BCL8* (Dyomin et al. 1997) and *MYLE* genes are located centromerically to the common deletion and within flanking repeats, respectively (Christian et al. 1998).

Although four paternally expressed genes have been identified within the PWS deletion region, no correlation between the loss of expression of a specific PWS region gene and the PWS phenotype has been shown. Furthermore, no affected individuals have been identified in whom a mutation affects the expression of only one PWS region gene, suggesting that PWS is a contiguous gene syndrome in which the loss of expression of several genes is required for full manifestation of the disorder. The PWS-AS common deletions span the 4-Mb interval between the flanking repeated sequences. However, the telomeric boundary for genes critical to PWS is now limited by a deletion-breakpoint marker (D15S174) that was identified in a family segregating an unusual submicroscopic deletion of proximal human chromosome 15 (Hamabe et al. 1991; Greger et al. 1993).

PWS mouse models have also helped to define a critical imprinting region. Comparisons with the mouse assume that the gene order is the same in both species, as has been reported (Gabriel et al. 1999; Ji et al. 1999). Deletions of the IC-equivalent region produce mice with failure to thrive (Yang et al. 1998). Mice with single gene deletions of *Zfp127, Snrpn,* and *Ipw* show no overt phenotype (Yang et al. 1998; Gerard et al. 1999). Two

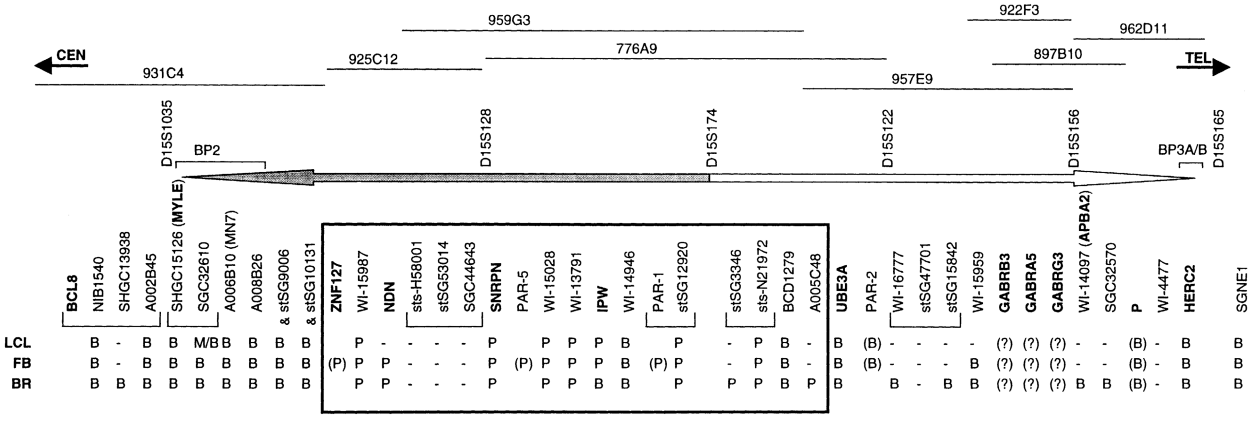

Figure 1 Map of the PWS-AS deletion region. The PWS-AS common-deletion region is marked by the large double-headed arrow, with the portion in gray denoting the PWS critical region. A minimal set of overlapping YAC clones (*top*) was used to localize ESTs (*middle*) within the deletion region. Genes are indicated in boldface type. STS (D15S) markers are noted below the YACs on the map. The imprinted expression profile for the ESTs and genes is indicated at the bottom. Previously reported results are in brackets; results from this study are not. Tissues studied were lymphoblast cell lines (LCL), fibroblast cell lines (FB) and brain (BR). Minus sign (−) = not expressed; blank = not done; question mark (?) = not certain, since contradictory results have been obtained by using different methods. Expression was detected in the RT-PCR assay as indicated: B = biparental; P = paternal; M/B = biased maternal. The relative order of markers in brackets was not determined. BP2, BP3A, and BP3B represent the repeated units that flank the common PWS-AS deletion region. The order of ESTs and markers within BP2 is according to Christian et al. (1999). These markers are repeated in BP3A and BP3B but are not shown on this map. The cluster of paternally expressed transcripts is boxed. ESTs stSG9006 and stSG10131 (denoted by ampersand [&]) were present on YAC 931C4, which places them in the interval between and including markers BCL8 and A008B26, but they have not been more finely localized and so are drawn next to A008B26.

Ndn-deficient mouse strains were recently generated by homologous recombination in ES cells. One necdin-deficient mouse strain (Gerard et al. 1999) is affected by failure to thrive and respiratory defects, which results in incompletely penetrant postnatal lethality on paternal transmission. *Ndn* is the first gene from the PWS deletion interval whose single mutation in mice demonstrates a phenotype with some aspects of PWS. The other necdin-deficient mouse strain (Tsai et al. 1999*a*) has no apparent phenotype, possibly because of strain-specific differences. A mouse with a radiation-induced deletion generated between *P* and *Ipw* does not produce a phenotype when paternally inherited (Johnson et al. 1995). However, a genetically engineered paternal deletion from *Snrpn* to *Ube3a* causes hypotonia, growth retardation, and partial lethality, which suggests the presence of a gene contributing to these aspects of PWS that is located between *Snrpn* and *Ipw* (Tsai et al. 1999*b*). Although it is possible that PWS mice do not exhibit the same phenotypic spectrum as humans, these cumulative results suggest the presence of an unidentified, paternally expressed gene in the critical PWS region whose loss of expression is responsible for the PWS phenotype. Alternatively, PWS may result from the absence of expression from several contiguous paternally expressed genes located in the centromeric part of the common-deletion region.

We set out to define the boundaries of the imprinted region through analysis of expressed sequence tags (ESTs [Boguski and Schuler 1995]). To address the possibility that additional imprinted genes reside within 15q11-q13, we have further analyzed a set of 40 ESTs that have been either physically mapped or radiation-hybrid mapped to chromosome 15q11-q13. By using a reverse transcriptase–PCR (RT-PCR) assay and physical mapping on a YAC contig, we have identified seven uncharacterized ESTs that are exclusively expressed from the paternal allele and one EST that is primarily maternally expressed in a tissue-specific manner. Our results suggest that $\geq$13 paternally expressed transcriptional units reside in the PWS critical region, 10 of which are centromeric to D15S174 and therefore may play a role in the PWS phenotype. The clustering of paternally expressed transcripts further suggests a strong regional control of imprinting.

Material and Methods

Chromosome 15 ESTs

The 40 chosen ESTs are listed below according to their mapping source, and their analysis is summarized in figure 2. The 27 unique ESTs used for mapping and imprinting analysis are underlined (fig. 1; table 1). Primers for these 27 ESTs were obtained from Research Genetics, Life Technologies, or the University of Alberta oligonucleotide synthesis facility. New primers were designed for BCD1279 (BCD1279-3F, 5′-GTTGATCCCTTTTTGCTCCA, and BCD1279-4R, 5′-GAAGCTGGCAATATGTCAACC). Sequence was unavailable for *BCL8* (R. Chaganti, personal communication). ESTs derived from the GeneMap'98 GB4 map were as follows: NIB1540, A002B45, SGC32610, WI-15987, BCD1279, stSG9006, stSG10131, stSG26368, sts-H58001, WI-15028, WI-13791, WI-15655, WI-14946, A009W43, stSG3346, A005C48, sts-N21972, SGC44643, stSG12920, stSG15842, WI-16777, stSG2525, sts-T16604, WI-6654, stSG9627, Cda0jb12, and WI-15959. stGDB:451595 mapped to two different chromosomes and was not analyzed further. ESTs derived from the GeneMap'99 GB4 map were as follows: stSG45529, stSG52513, WIAF-831, stSG53014, WIAF-778, and stSG47701. ESTs derived from an integrated YAC contig (Christian et al. 1998) were as follows:

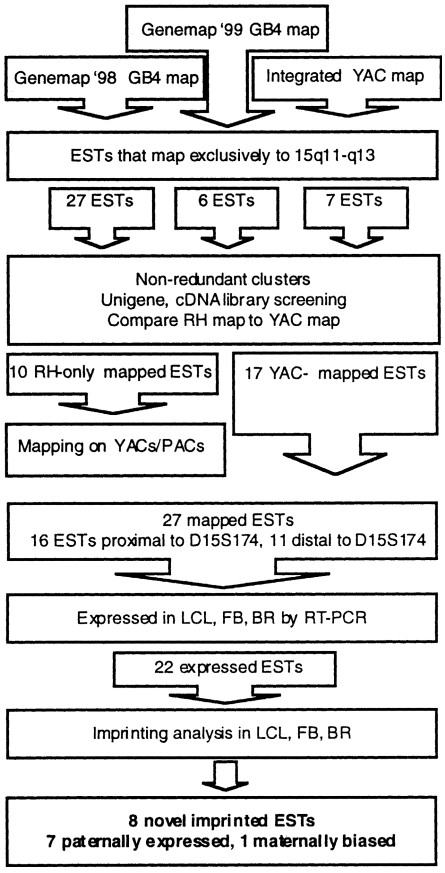

Figure 2 Flow chart illustrating the selection process for ESTs in the 15q11-q13 region. The source of the ESTs is indicated at the top. The process by which ESTs were sorted for redundancy, placed on the physical map, and tested for expression and imprinted is represented, with the resulting identification of eight novel imprinted transcripts indicated at the bottom of the flowchart.

Table 1

Transcription Units and Genes in 15q11-q13

EST or Gene	UniGene Cluster	Map Source
NIB1540, stSG45529, stSG52513	Hs.83724	YAC
SHGC13938	…	YAC
A002B45	Hs.8177	YAC
D15S1035	STS marker	YAC
MYLE, SHGC15126	Hs.11902	YAC
SGC32610	Hs.126738	YAC
MN7, A006B10	Hs.174087	YAC
A008B26	Hs.15543	YAC
ZNF127	Hs.72964	YAC
WI15987, WIAF831	Hs.138750	YAC
NDN, NDN5F/6R	Hs.50130	YAC
stSG53014	Hs.180992	RH
BCD1279-3F/4R	…	RH
stSG9006	Hs.931125	RH
stSG10131, stSG26368	Hs.15548	RH
sts-H58001	Hs.37456	RH
D15S128	STS marker	YAC
SNRPN, SGC31492, R99003	Hs.48375, Hs.131891	YAC
PAR-5	Hs.179987	YAC
WI-15028	Hs.31797	YAC
WI-13791, WI-15655	Hs.22543, Hs.23231	YAC
IPW, IPW-60A/60B	Hs.5022	YAC
WI-14946, A009W43, WIAF-778	…	YAC
PAR-1	…	YAC
stSG3346	…	YAC
A005C48	…	YAC
UBE3A, WI-6519, stSG3525, WI-13724	Hs.180686	YAC
PAR-2	…	YAC
sts-N21972	Hs.43052	RH
SGC44643	…	RH
stSG12920	…	RH
stSG47701	Hs.163845	RH
stSG15842	…	RH
D15S122	STS marker	YAC
WI-16777	…	YAC
WI-15959	Hs.143997	YAC
GABRB3	Hs.1440	YAC
GABRA5	Hs.24969	YAC
GABRG3	…	YAC
D15S156	STS marker	YAC
APBA2, WI-14097	Hs.26468	YAC
SGC32570	Hs.167626	YAC
P	Hs.82027	YAC
WI-4477	…	YAC
D15S165	STS marker	YAC
SGNE1, SGNE1-1F/2R	Hs.2265	RH

NOTE.—ESTs were selected from GeneMap'98, GeneMap'99, and an integrated YAC contig (Christian et al. 1998). ESTs were placed into the correct physical mapping interval with respect to the STS markers D15S1035, D15S128, D15S122, D15S156, and D15S165 by YAC mapping (Y [Christian et al. 1998]). Otherwise, ESTs were placed in the appropriate interval according to the radiation-hybrid (RH) mapping data, but they were not ordered with respect to each other in this table. Some ESTs were found to be part of the same transcriptional unit on the basis of database searches but were not previously included in the UniGene cluster. Previously known genes (italicized) and transcripts are also included. ESTs or primers used for RT-PCR analysis are underlined. The UniGene cluster information was obtained through a search of the UniGene database. ESTs indicated with an ellipsis (…) have not yet been assigned to a UniGene cluster.

SHGC13938, SHGC15126, SHGC17218, A008B26, WI-14097, SGC32570, and WI-4477. SHGC17218 mapped to two different chromosomes and was not analyzed further.

Unique ESTs for known genes were as follows: A006B10 (*MN7*), SGC35648 (*ZNF127*), NDN-5F and NDN-6R (*NDN* [MacDonald and Wevrick 1997]), SGC31492, R99003 (*SNRPN*), IPW-60A and IPW-60B (*IPW* [Wevrick et al. 1994]), WI-6519 (*UBE3A*), and SGNE1–1F, 5′-TAGGCCTCAGCATGGCTTAT, and SGNE1-2R, 5′-CCAAGGGCTGGGTGAACTAC (*SGNE1*).

Cell Lines and Tissues

Chromosome 15–deletion lymphoblast cell lines (PWS, GM09024B; and AS, GM11515) and control fibroblast (GM00909) cell lines were obtained from the NIGMS Human Genetic Mutant Cell Repository. A chromosome 15–deletion fibroblast cell line, PWS-deletion brain sample (1889), and control cerebellum sample (2144) were obtained from the University of Miami Brain and Tissue Bank for Developmental Disorders. The AS frontal cortex sample (CBTB 293) was generously provided by Dr. Marc Lalande, University of Connecticut. Control lymphoblast cell lines were obtained from Dr. Ian MacDonald, University of Alberta. AS fibroblasts were generously provided by Dr. Arthur Beaudet, Baylor College of Medicine.

RT-PCR Analysis

Trizol reagent (Life Technologies) was used to isolate RNA from cell lines and tissues. RNA was treated with 1 U of RQ1 RNase-Free DNase (Promega). RT was performed with 100 ng of random primer $p(dN)_6$, 200 U of Superscript II reverse transcriptase, 4 μl 5 × first strand buffer, 10 mM DTT, and 0.5 mM dNTP(Life Technologies). RT reactions were carried out in an MJ Research PTC 100 or PTC 200 thermal cycler, with incubation at 42°C for 45-min followed by a 95°C incubation for 5 min. PCR was performed in 20-μl reactions containing 0.5 U of recombinant *Taq* DNA polymerase, 1.0–3.0 mM $MgCl_2$, 0.2 mM dNTP (Life Technologies), 0.5 mM primers, and 100 ng of cDNA or 50 ng of control genomic DNA. A separate reaction with no RT was performed, to ensure that no genomic DNA contaminated the RT reaction. The reactions were performed in an MJ Research PTC 100 or PTC 200 with an initial denaturation at 95°C for 5 min, followed by 30–35 cycles of denaturation at 95°C for 30 s, annealing at 50–62°C for 30 s, extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min. The reaction products were resolved on 2% agarose gels, stained with ethidium bromide, and visualized with an ImageMaster VDS gel documentation system (Amersham/Pharmacia

Biotech). All RT-PCR experiments were repeated at least twice.

Large-Insert Clones

YAC clones 931C4, 925C12, 959G3, 776A9, 957E9, 922F3, 897B10, and 962D11 were obtained from Research Genetics or from the Medical Research Council Genome Resource Facility (Hospital for Sick Children, Toronto). YAC DNA was isolated by lysis of cells with 0.5-mm–diameter acid-washed glass beads (Sigma-Aldrich Canada), followed by treatment with 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 200 μl of phenol/chloroform/isoamyl alcohol (25:24:21) and precipitation with ammonium acetate and ethanol (Scherer and Tsui 1991). YAC DNA (50 ng) was then used directly for PCR. PAC clones pDJ50I2, pDJ121M14, pDJ121D5, and pDJ134I14 were obtained from the Genome Resource Facility, and DNA was prepared by alkaline lysis.

Results

Selection of Expressed Sequences: EST Contig Building and Library Screening

The predicted critical region for PWS genes is centromeric to D15S174. To focus on this region, while taking into account the imprecision of radiation-hybrid mapping, ESTs were initially chosen from the region between the markers D15S1035 and D15S156 that flank the smaller PWS-critical region (fig. 1; table 1). All ESTs localized to this radiation-hybrid mapping interval in GeneMap'98 were analyzed, and those that corresponded to known genes were excluded. Two ESTs were present on more than one chromosome by radiation-hybrid analysis and were not analyzed. Of the remaining EST set, 27 ESTs were selected for analysis. Six ESTs identified by similar analysis of GeneMap'99 were added. During the course of this analysis, a YAC map of the PWS-AS deletion region was published (Christian et al. 1998), from which we selected 7 additional ESTs, for a total of 40 ESTs. The centromeric part of the PWS-AS deletion region was emphasized in our EST selection, so that not all ESTs from the telomeric region were included in this study. We then analyzed the information in UniGene, a system for assigning sequences to nonredundant clusters, each of which should represent a separate transcriptional unit (Boguski and Schuler 1995). A set of 28 ESTs remained after this step. A cDNA clone spanning the UniGene clusters for WI-13791 and WI-15655 was identified by screening of a heart cDNA library (data not shown), which indicates that these clusters are part of the same transcription unit. After database analysis and grouping into EST clusters, a minimum set of 27 unique ESTs that did not correspond to known genes remained; this set was further analyzed.

Physical Mapping of Unique ESTs

Physical maps of the PWS deletion region and its flanking repeats have been generated on the basis of large-insert DNA clones (Christian et al. 1998, 1999; Amos-Landgraf et al. 1999). One group of ESTs, represented by the ESTs SGC32610, SHGC-15126 (*MYLE*), SHGC17218, A008B26, and A006B10 (MN7), had previously been shown to be present in at least two copies, located at the centromeric and telomeric breakpoints of the common-deletion region (Christian et al. 1999). Of the 27 unique ESTs that we selected, 10 had not been physically mapped but were predicted to be in or near the PWS deletion interval on the basis of their location between D15S1035 and D15S122 on the radiation-hybrid map (table 1). To place these ESTs on the physical map, DNA was isolated from a series of overlapping YACs spanning the PWS common-deletion region and tested for content of known markers to confirm their identity (data not shown). YAC DNA was then tested by PCR with EST primers, placing the 10 ESTs on the physical map (fig. 1). Three ESTs, SGC44643, sts-H58001, and stSG53014, are located in the interval between *NDN* and *SNRPN*, a region important for PWS but not previously known to contain any genes (fig. 1). Two ESTs (stSG9006 and stG10131) were located proximally on YAC931C4 but could not be more finely mapped. Three ESTs (stSG12920, sts-N21972, and BCD1279) were located on the overlapping YACs 959G3 and 776A9. These ESTs were more finely mapped with PAC clones from this region that contain reference STS markers (data not shown).

Imprinting Status of Expressed Sequences

The PWS-AS common-deletion region contains genes expressed exclusively from the paternal allele, one gene expressed predominantly from the maternal allele in a tissue-specific fashion, and genes expressed from both alleles. Three cDNA fragments located within the common-deletion region were reported to be paternally expressed (PAR-5 and PAR-1 [Sutcliffe et al. 1994]) or biallelically expressed (PAR-2 [Nakao et al. 1994]). However, the allelic expression pattern of other ESTs mapped to the PWS-AS common-deletion region had not been assessed. To address this, an RT-PCR assay that detected parent-of-origin–specific expression was designed. RNA was isolated from tissues or cell lines from a control individual, a person with PWS with a paternal 15q11-q13 deletion, or a person with AS with a maternal 15q11-q13 deletion. Because of deletions of the imprinting-control element in the PWS or AS patients, we pre-

dicted that genes in 15q11-q13 that are exclusively paternally expressed would show no expression in the PWS samples, as previously demonstrated for *SNRPN* (Wevrick and Francke 1996). Similarly, we hypothesized that genes that are exclusively maternally expressed would show no expression in the AS-derived samples. To test this hypothesis, RNA samples from lymphoblasts and fibroblasts and brain from control subjects and persons with PWS or AS were tested by RT-PCR with gene primers from the 15q11-q13 region. As expected, the known imprinted, paternally expressed *NDN* and *SNRPN* genes were active only in AS and control samples, with no product visible in the PWS samples (fig. 3). *NDN* is not expressed in lymphoblasts, so this cell type could not be assessed for imprinting. Surprisingly, *IPW* was active in control, PWS, and AS brain but was restricted to control and AS samples in fibroblasts and lymphoblasts, suggesting that its imprinting is tissue-specific (fig. 3). The *MN7* transcripts were equally active regardless of the parental origin of the allele or alleles present. Expression analyses had previously shown *UBE3A* to have biallelic expression in fibroblasts and lymphoblasts (Nakao et al. 1994) and a bias toward maternal expression in brain (Rougeulle et al. 1997; Vu and Hoffman 1997). In our nonquantitative assay, expression appeared to be biallelic in all tissues, including brain (fig. 3). These results validated the RT-PCR assay, although no maternally expressed gene was available as a control for maternal-only expression.

We then tested all 27 ESTs for expression in control RNA samples taken from lymphoblasts, fibroblasts, and brain. Five ESTs (SGC44643, WI-4477, stSG53014, stSG47701, and sts-H58001) were eliminated from further analysis because their transcripts could not be detected by RT-PCR. The remaining 22 unique ESTs showed expression in at least one tissue source, so RT-PCR was performed on control, PWS, and AS RNAs (figs. 1 and 3). From these results, the ESTs could be classified as having biparental expression (14 ESTs), paternal-only expression (7 ESTs), or maternal-biased expression (1 EST) in at least one tissue source. Of the seven new paternally expressed transcriptional units identified, four localize to the PWS critical region between D15S1035 and D15S174 and three are located just telomeric to D15S174 but centromeric to *UBE3A* (fig. 1). Two apparently biallelically expressed transcripts, represented by ESTs WI-14946 and BCD1279, are located among the set of paternally expressed transcripts. With one exception, all ESTs located centromeric to the deletion or within the flanking repeated regions were biparentally expressed. There are at least two copies of most of the ESTs in the breakpoint repeats (Christian et al. 1999), and our assay does not distinguish among these highly similar copies. It is therefore likely

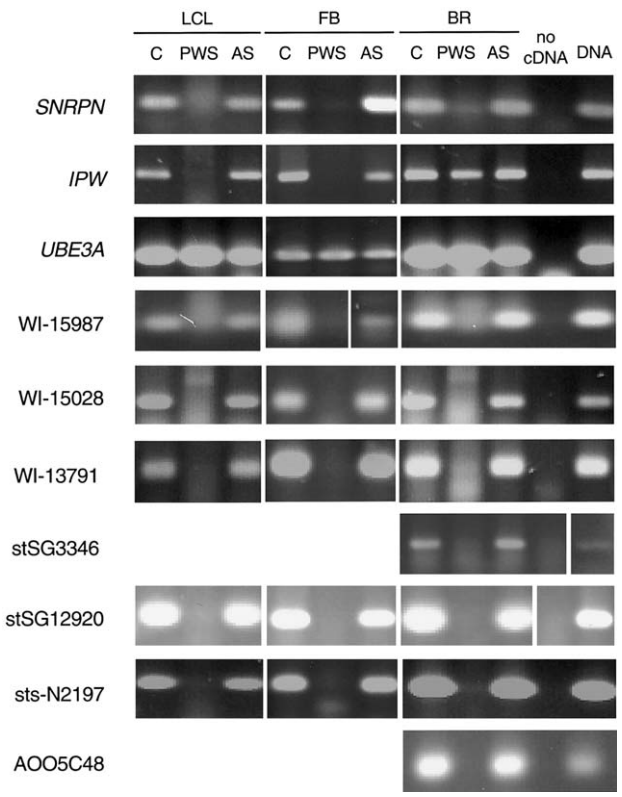

Figure 3 Imprinting status of genes and ESTs. RT-PCR was performed on RNA derived from lymphoblasts (LCL), fibroblasts (FB), and brain (BR) from control (C), deletion PWS (PWS), and deletion AS (AS) individuals. The second-to-last lane shows a PCR amplification with no cDNA added, and the last lane shows amplification with human genomic DNA. Negative control PCR reactions with no RT were performed but are not shown. The ESTs stSG3346 and A005C48 are not expressed in lymphoblasts or fibroblasts.

that the apparently biallelic RT-PCR products for ESTs within the breakpoint region (SGC32610, SHGC-15126, A006B10 [MN7], and A008B26) represent amplification from transcripts derived from multiple loci. This could mask a monoallelic expression profile from one of these loci. Surprisingly, the EST SGC32610, located within the breakpoint repeat, displayed maternal-biased expression in lymphoblasts, with reproducibly reduced expression evident in lymphoblasts from two different AS individuals. However, given the nonquantitative nature of this assay, further experiments are necessary to conclude definitively that SGC32610 is imprinted. One final gene, *SGNE1*, is located on chromosome 15 distal to the PWS deletion (Mattei et al. 1990) and was chosen for study because previous studies had indicated possible changes in protein level in PWS brain (Graham et al. 1992; Gabreels et al. 1994, 1998). However, the *SGNE1* transcript was expressed from both alleles in our assay.

Expression Profile of Imprinted ESTs

Our RT-PCR and mapping analyses indicated that seven newly identified paternally expressed ESTs are located within the ~4-Mb PWS-AS common-deletion region (see box in fig. 1). In addition, one EST that appears to show maternally biased expression is located within the breakpoint repeats. These ESTs are represented among cDNAs that have been identified as part of the dbEST portion of the Human Genome Project (Boguski et al. 1993) and have been grouped into UniGene clusters. Each UniGene cluster contains sequences that represent a unique gene; it also contains related information, such as the tissue types in which the gene is expressed. To estimate the expression level and range of tissue expression of the cDNAs represented by each EST, the UniGene clusters to which each imprinted gene or EST belongs were examined (table 2). In two cases (stSG12920 and A005C48), cDNA clones linked to the EST markers in GeneMap'98 were used because no UniGene cluster had been assigned to this EST. No cDNA clones were available in dbEST for PAR-1. The number of cDNA clones in each UniGene cluster gives a relative estimate of the gene's expression level. Each cDNA was counted only once in the analysis, even if both the 5′ end and the 3′ end were present in the UniGene cluster. Analysis of the UniGene clusters for *SNRPN*, *NDN*, *ZNF127, IPW,* and *UBE3A* (table 2) revealed a range of tissue expression for each gene. In comparison, a more limited range of tissue expression was seen for the newly described imprinted ESTs and PAR-5, a previously identified paternally expressed cDNA fragment. By use of the moderately expressed *SNRPN* gene as a comparison, the eight new imprinted transcription units are predicted to be expressed at very low levels in the tissues commonly used for generation of ESTs. The UniGene clusters for ESTs that do not correspond to known genes were also examined for coding potential through sequence analysis (BLASTX), but no protein-coding regions or significant potential open reading frames were found.

Discussion

In this report, we have identified a set of transcripts in the PWS-AS deletion region and determined the genomic imprinting status for each. We have identified eight novel imprinted transcription units within the PWS-AS deletion region. Among these, four new paternally expressed ESTs are located within the PWS critical region, as defined by the markers D15S1035 and D15S174 (Greger et al. 1993). If the gene order is conserved in mouse and the ESTs represent genes conserved between human and mouse, then only the three genes represented by WI-15987, WI-15028, and WI-13791 are inside the critical region, as defined by radiation-induced deletions in mouse (Johnson et al. 1995). The eight new imprinted transcription units generate lower-level transcripts than the previously identified PWS-region genes, by use of analysis of the number of cDNAs represented in UniGene and dbEST. Other developmentally important genes are expressed at low levels in fetal and adult tissues but are nonetheless essential at critical developmental stages and may cause abnormal development when absent (Odent et al. 1999). By RT-PCR, transcripts associated with all seven paternally expressed ESTs could be detected in brain, the major organ of involvement in PWS (fig. 1). Furthermore, five of the imprinted ESTs are represented in cDNA libraries derived from brain (table 2). Thus, the genes represented by these ESTs should be included with previously identified paternally expressed genes as potentially responsible for the manifestations of PWS.

The phenotype of AS is thought to be primarily due to the absence of *UBE3A* expression, since a subset of AS patients carry mutations only in the *UBE3A* gene. However, there is evidence to suggest that deletion cases are more severely affected than nondeletion ones (Rougeulle and Lalande 1998). The EST SGC32610, located in the repeats that flank the PWS-AS deletion, appears to be preferentially maternally expressed in lymphoblasts. This is similar to previous results for *UBE3A,* which showed incomplete imprinting in brain, perhaps indicating that control of maternal-specific expression is less restricted than that for paternal-specific expression in the PWS-AS region. The transcript represented by SGC32610 is the first example of a gene that could modify the AS phenotype through loss of expression in deletion and IC AS cases. Overexpression of the gene represented by the EST SGC32610 may also be implicated in the phenotype of patients with additional maternally derived copies of chromosome 15q11-q13 (Browne et al. 1997; Huang et al. 1997). A complete transcript map will aid the identification of genes for other disorders associated with 15q11-q13, including spastic paraplegia, obsessive-compulsive disorder, bipolar illness, and autism (Christian et al. 1998). Further investigation of the genes associated with the imprinted ESTs, and their imprinting status in other tissues and developmental stages, is clearly needed to understand their possible roles in the etiology of disorders involving 15q11-q13.

The EST RT-PCR assay described here is an extension of an *SNRPN* RT-PCR assay previously proposed as a rapid diagnostic test for PWS in small blood samples (Wevrick and Francke 1996). A similar assay has also been used to analyze the X-inactivation status of a number of transcribed sequences across the Xp11 region of the human X chromosome (Miller and Willard 1998). Although RT-PCR is very sensitive for the detection of

Table 2

Estimate of Expression Levels for Imprinted Transcription Units

	PAT												MAT	
	ZNF127	WI-15987	NDN	SNRPN	PAR-5	WI-15028	WI-13791	IPW	stSG3346	stSG12920	sts-N21972	A005C48	SGC32610	UBE3A
Fetal tissues:														
Brain	…	…	Yes	Yes	Yes	…	Yes	Yes	…	…	…	…	…	Yes
Cochlea	…	…	Yes	…	…	…	…	…	…	…	Yes	…	…	…
Heart	…	…	…	Yes	…	…	Yes	Yes	…	…	…	…	…	…
Liver spleen	…	…	…	Yes	…	…	…	Yes	…	Yes	…	…	Yes	Yes
Embryo	…	…	Yes	Yes	…	…	…	…	…	…	…	…	…	Yes
Adult tissues:														
Adrenal gland	…	…	Yes	Yes	…	…	…	Yes	…	…	…	…	…	…
Brain	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	…	…	Yes	…	Yes
Bone	…	…	…	…	…	…	…	…	…	…	…	…	…	Yes
Breast	…	…	…	Yes	…	…	…	…	…	…	…	…	…	…
Colon	…	…	…	Yes	…	…	…	…	…	…	…	…	…	Yes
Eye, retina, fovea	…	…	Yes	Yes	Yes	…	Yes	…	…	…	…	…	…	Yes
Gallbladder	…	…	…	Yes	…	…	…	…	…	…	…	…	…	…
Germ cell, testis	…	…	Yes	Yes	Yes	…	…	Yes	…	…	…	…	…	Yes
Heart/aorta	…	…	Yes	Yes	Yes	…	Yes	…	…	…	…	…	…	Yes
Kidney	…	…	Yes	Yes	…	…	Yes	Yes	…	…	…	…	…	Yes
Liver	…	…	…	Yes	…	…	…	…	…	…	…	…	…	…
Lung	…	Yes	Yes	Yes	Yes	…	Yes	Yes	…	…	…	…	…	Yes
Muscle	…	…	Yes	Yes	…	…	Yes	…	…	…	…	…	…	Yes
Ovary	…	…	…	Yes	…	…	…	…	…	…	…	…	…	Yes
Pancreas	…	…	Yes	Yes	…	…	…	…	…	…	…	…	…	Yes
Parathyroid	…	…	…	…	…	…	…	Yes	…	…	…	…	…	Yes
Placenta	…	…	…	…	…	…	…	…	…	…	…	…	…	Yes
Prostate	…	…	Yes	Yes	…	…	…	…	…	…	…	…	…	Yes
Skin	…	…	…	…	…	…	…	…	…	…	…	…	…	Yes
Stomach	…	…	…	Yes	…	…	Yes	Yes	…	…	…	…	…	Yes
Synovial membrane	…	…	Yes	…	…	…	…	…	…	…	…	…	…	…
T cells, blood cells	Yes	…	…	Yes	…	…	…	Yes	…	…	…	…	…	…
Thyroid	…	…	…	Yes	…	…	…	…	…	…	…	…	…	Yes
Tonsil	…	…	…	…	…	…	Yes	…	…	…	…	…	…	Yes
Uterus	…	Yes	Yes	Yes	…	…	…	Yes	…	…	…	…	…	Yes
No. of cDNA clones	3	4	41	126	7	2	15	36	1	1	1	1	3	142

NOTE.—Representation in UniGene and dbEST was used to estimate the expression levels and tissue distribution of paternally expressed (PAT) and biased maternal expressed (MAT) transcripts and genes. In adult tissues, brain includes cDNA libraries constructed from infant brain, central nervous system, brain, multiple sclerosis lesion, cerebellum, NT-2 cells, and Schwannoma tumor. "Yes" indicates that cDNA from that tissue is in the UniGene cluster. Ellipses (…) = not present in tissue.

low-level transcripts, we have used it in a nonquantitative fashion. Thus, imprinted transcripts that show unequal allelic expression appear to have biallelic expression in this assay. Furthermore, transcripts that are imprinted in a tissue-specific manner may not reveal monoallelic expression in the limited tissue types tested in our assay. For example, in the Beckwith-Wiedemann syndrome region, transcripts have shown monoallelic expression during embryogenesis, with gradual loss of imprinting in adult tissues (Dao et al. 1998). Finally, some imprinted genes are associated with antisense transcripts, which may or may not have the same imprinting profile (Rougeulle et al. 1998; Jong et al. 1999; Lee et al. 1999*a*, 1999*c*; Mitsuya et al. 1999). Imprinted genes that are associated with antisense transcripts could appear biallelic in the RT-PCR assay if the EST primers also amplify the antisense transcript. This can be resolved by performance of a strand-specific RT followed by PCR. In summary, we have demonstrated that the EST RT-PCR assay is an effective tool to identify imprinted transcripts, although it may falsely identify some imprinted transcripts as having biallelic expression.

We also provide further evidence for the clustering of imprinted genes. Analysis of our data strongly suggests that the imprinted and paternally expressed region is bounded on the centromeric side by the centromeric repeat region near D15S1035, since all transcripts centromeric to this marker are apparently expressed in a biallelic manner. On the telomeric side, the paternally expressed domain is bounded by *UBE3A*, with all transcripts distal to this gene expressed from both alleles. EST SGC32610, located within the breakpoint repeats, appears to show a bias toward maternal expression, although the apparent reduction in expression from the maternal allele must be confirmed by a quantitative assay on additional AS samples. These data are consistent with prior observations that imprinted genes tend to occur in clusters in both human and mouse genomes (Reik and Walter 1998). Two transcripts (WI-14946 and BCD1279) located among the cluster of paternally expressed transcripts are biallelically expressed. Studies from the imprinted chromosome 11p15 region have shown that biallelically expressed genes can be found within a group of imprinted genes (Lee et al. 1999*b*). Alternatively, these transcripts may show tissue-specific imprinting that is undetected in the limited number of tissues tested in our assay, or they may have undetected unequal allelic expression.

Both gene-specific and regional cues have been implicated to explain the clustering of imprinted genes and the common occurrence of oppositely imprinted genes in the same genomic vicinity (Barlow 1997; Constancia et al. 1998). The clustering of imprinted genes and a need for oppositely imprinted genes in the same chromosomal vicinity may indicate a mechanism for imprinting control at the gamete stage. In the PWS region, deletion of an imprinting-control element results in failure of imprinted genes to adopt the proper epigenetic imprint during gametogenesis (Horsthemke 1997). These deletions, when paternally inherited, result in PWS because of the loss of expression of genes that are normally expressed from the paternal allele. The range over which genes are controlled by the IC is unknown but is not necessarily confined to the common-deletion interval, as defined by breakpoint repeats that predispose to deletion. The identification of an imprinted gene outside these boundaries would implicate either a second imprinting-control element or would point to a model whereby the *SNRPN*-associated IC could skip over a cluster of nonimprinted genes and act on a gene beyond this cluster. More complete understanding of the range of action of the IC awaits further analysis of imprinting of genes on proximal chromosome 15q.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for PWS [MIM 176270] and AS [MIM 105830])
BLASTX, http://www.ncbi.nlm.nih.gov/blast/blast.cgi
GeneMap'98, http://www.ncbi.nlm.nih.gov/genemap98/
GeneMap'99, http://www.ncbi.nlm.nih.gov/genemap99/
UniGene, http://www.ncbi.nlm.nih.gov/Schuler/UniGene/index.html

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